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DNA synthesis blocking lesions induced by singlet oxygen are targeted to deoxyguanosines

Denise T.Ribeiro[1,2], François Bourre[3], Alain Sarasin[3], Paolo Di Mascio[1,+] and Carlos F.M.Menck[1]*

[1]Departamento de Biologia, Instituto de Biociências, CP No. 11461, Universidade de São Paulo, SP 05499, [2]Departamento de Biologia Celular, Instituto de Biologia, Universidade de Brasília, Brasília, DF, Brazil and [3]Laboratoire de Génétique Moleculaire, Institut de Recherches Scientifiques sur le Cancer, Centre National de la Recherche Scientifique, BP No. 08, 94801, Villejuif, France

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ABSTRACT

***In vitro* DNA synthesis on single stranded templates damaged by singlet oxygen was investigated in the *supF* tRNA gene sequence, using several DNA polymerases. Singlet oxygen was generated by the thermal decomposition of the water soluble with the endoperoxide of disodium 3,3'-(1,4-naphthylidene) dipropionate ($NDPO_2$). The data demonstrated that damage at deoxyguanosine residues interrupts DNA polymerization. Modified T7 phage and *Thermus aquaticus* DNA polymerases were found to synthesize DNA fragments which terminated opposite deoxyguanosine, while T4 phage DNA polymerase and avian myeloblast virus reverse transcriptase were blocked one nucleotide 3' to deoxyguanosine positions on the template. DNA polymerase I (Klenow fragment) from *Escherichia coli* was inhibited at both positions, before and at the putative damaged sites. The blocking lesions, induced by 5 mM $NDPO_2$, were estimated to be approximately 1.5 per 260 nucleotides, corresponding to 2% of deoxyguanosines. The distribution of lesions in the *supF* gene did not reveal any specific sequence context which showed distinct susceptibility to the attack of singlet oxygen.**

INTRODUCTION

Singlet oxygen ($^1O_2$) is a very reactive species with great potential for causing biological hazards, including genomic damage. This excited molecule has a relatively long lifetime and high diffusion rate in aqueous solution (1), which increase its reactivity with macromolecules having high electron density. Thus, there is a growing interest in the deleterious action of $^1O_2$ on DNA and its mutagenic consequences (2).

It is known that free guanine is the DNA residue preferentially oxidized by $^1O_2$ (3,4). The $^1O_2$ reactivity with DNA molecules is much lower than with free guanine nucleotides in solution (4), although, there is clear evidence that it can damage DNA. Breaks in the phosphodiester backbone and alkali-labile sites (5,6,7) were observed after exposure of DNA to excited photosensitizers, which may produce $^1O_2$. Di Mascio et al. (8) demonstrated the induction of single strand breaks by $^1O_2$ generated by three different sources. Double strand breaks were detected in DNA exposed to $^1O_2$ at high concentrations (9). It was also shown that $^1O_2$ is more reactive with single stranded (ssDNA) than double stranded DNA (dsDNA), yielding a higher number of breaks in the phosphodiester chain (10).

The specific reactivity of $^1O_2$ with nucleotides in DNA has been investigated. Piperidine labile sites at deoxyguanosine (dG) residues were observed in DNA exposed to photosensitized methylene blue (11,12). Piette and Moore (13) observed termination of DNA synthesis by *E.coli* DNA polymerase I at nucleotides preceding dGs, on a DNA template treated with proflavine and light, suggesting the presence of lesions at this base. Part of these lesions are due to the action of $^1O_2$ produced by excited proflavine, as concluded from the effects of sodium azide (NaN_3) and deuterated water (D_2O) on the $^1O_2$ reaction efficiency (14). However, it should be noted that proflavine binds to DNA and yields photoproducts, other than $^1O_2$, capable of interacting with DNA, which may result in biased data. Recently, Floyd et al. (15) have shown the production of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in DNA irradiated in the presence of methylene blue. The enhancing effect of D_2O on this reaction indicated that $^1O_2$ mediates the formation of 8-OH-dG. This kind of damage was also detected after treatment of free nucleotide with the endoperoxide of disodium 3,3'-(1,4-naphthylidene) dipropionate ($NDPO_2$) (16), a highly specific source of $^1O_2$ (17). The biological importance of the $^1O_2$-induced lesions was demonstrated by the mutagenicity of $^1O_2$ in bacteria (9,18) and in mammalian cells (9,10).

In this work, the sequence specificity of lesions induced in ssDNA treated with $^1O_2$ generated by the thermodissociation of

* To whom correspondence should be addressed

+ Present address: Instituto de Química, CP 20780, USP, São Paulo, SP 05499, Brazil

$NDPO_2$ was analyzed. This analysis was performed according to the method of Moore and Strauss (19), who found that lesions in the template may block *in vitro* DNA synthesis by DNA polymerase I from *E.coli*. The data presented here demonstrate that the damaging action of $^1O_2$ on DNA is targeted to dGs, i.e., depending on the polymerase used, DNA synthesis is blocked one base 3′ to or at the position of a dG residue on the damaged template. Moreover, in the sequence studied, the *supF* tRNA gene, no influence of the flanking bases on the formation of blocking sites by $^1O_2$ was detected.

EXPERIMENTAL PROCEDURES

Materials

Deoxyribonucleoside triphosphates were obtained from SIGMA or United States Biochemical Corporation; (^{35}S)dATP was from Amersham. DNA polymerases of T4 phage (T4 DNA polymerase), of *Thermus aquaticus* (Taq) and DNA polymerase I, Klenow fragment, were purchased from New England Biolabs; the modified polymerase of T7 phage (sequenase), from United States Biochemical Corporation and avian myeloblastosis virus reverse transcriptase (AMV-RT), from Promega.

Plasmid and ssDNA preparation

The plasmid used, πSVPC13FIA, has been previously described in detail (10); it carries the *supF* tRNA gene sequence which was employed as template in the DNA synthesis assays. It also has the *f1* phage origin of replication, which allows the plasmid to enter the phage replication mode within permissive bacteria after infection with a helper phage. The ssDNA was prepared from the JM105 strain (20) carrying the plasmid by the procedure described in Sambrook et al. (21), using M13K07 (Pharmacia PL, Biochemical Inc.) as helper phage.

DNA treatment with $^1O_2$

Singlet oxygen was produced by the thermodissociation of the water soluble $NDPO_2$ yielding 3,3′-(1,4-naphthylidene) dipropionate (NDP) and molecular oxygen, half in the triplet state and half in the excited singlet state (17). $NDPO_2$ concentration was determined spectrophotometrically (288 nm). ssDNA samples (2 μg/ 200 μl) were incubated with $NDPO_2$, in 50 mM sodium phosphate buffer in D_2O, pD 7.4, at 37°C, for 90 min. After treatment, 0.6 volume of a solution of 20% PEG and 2.5 M NaCl was added to the samples and they were kept for one hour in ice. The DNA was then centrifuged, resuspended in 20 μl of 0.2 N NaOH and precipitated with 0.4 volume of 5 M ammonium acetate and ethanol. These steps preceded annealing with primer in order to purify the DNA for the polymerase reactions.

DNA polymerase assays

The treated ssDNA (1 μg) was annealed with FM10 primer (5′-CTAGTTCGATGATTAA-3′), which is complementary to the vector, adjacent to the *supF* gene. Hybridization was performed in 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$ and 50 mM NaCl, at 65°C for 5 min, followed by gentle cooling to room temperature. The molar ratio of primer to ss plasmid DNA was 10 to 1. The polymerase reactions contained 0.3 μM each of (^{35}S)dATP (1000 Ci/mmol), dGTP, dCTP and dTTP in standard buffer (26.5 mM Tris-HCl pH 7.5, 13.3 mM $MgCl_2$, 16.6 mM NaCl and 6.7 mM DTT) for the labelling reaction and, alternatively, one of each enzyme: 1 U T4 DNA polymerase, 2 U Klenow fragment, 2.5 U Taq DNA polymerase, 9.5 U AMV-RT or 3.3 U sequenase. For the elongation reaction, a mixture of 83 μM of the four dNTPs was added. The labelling reaction for the sequenase and T4 DNA polymerase lasted 2 min at room temperature and for the elongation 5 min at 37°C. For Klenow fragment the first step took 15 min at room temperature and the second 12 min, at 37°C. For AMV-RT and Taq DNA polymerase, the labelling reaction was performed for 10 min, at 42°C, and the elongation 10 min, at 42°C (AMV-RT) or 70°C (Taq DNA polymerase). The reactions were terminated by addition of 0.6 volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The DNA sequencing reactions were performed in parallel using sequenase and the Sanger method (21).

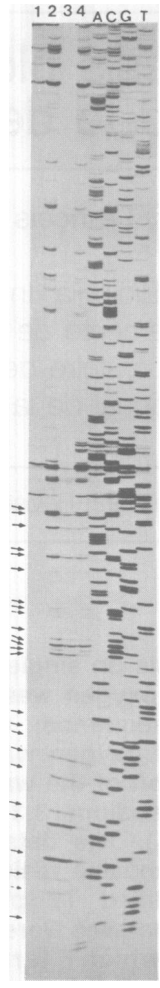

Figure 1. DNA synthesis of $NDPO_2$-treated ssDNA template by sequenase. The template was treated with $NDPO_2$ at the following concentrations: (1) 0 mM; (2) 1 mM; (3) 10 mM, and (4) 10 mM plus 1 mM NaN_3. Lanes A, C, G and T correspond to the sequence of the synthesized strand. Arrows indicate the positions of dGs at the template strand.

Product Analysis

Heat denaturated products of the various polymerization reactions were loaded and electrophoresed on standard denaturating (7 M urea) 8% polyacrylamide gels. After drying, the gels were autoradiographed with a Kodak X OMAT-K film for 2 days. The autoradiograms were scanned with Ultrascan XL-Pharmacia LKB

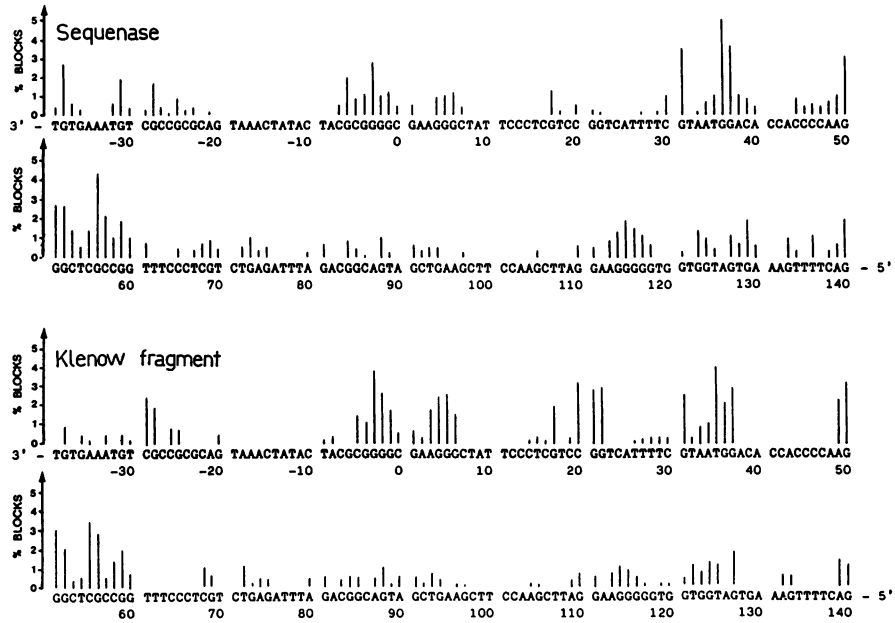

Figure 2. Distribution of DNA synthesis $^1O_2$-induced blocking sites on the *supF* gene. The ssDNA template was treated with 1 mM (for sequenase) or 5 mM (for Klenow fragment) and the blocking site intensities determined by densitometry (indicated by each bar) from experiments as those shown in Figure 1. Numbers are relative to the beginning of the tRNA *supF* transcription.

densitometer. The number of blocking lesions was estimated considering that the molecules with sequences larger than 260 bases were synthesized from templates with no lesions. The average number of lesions per 260 bases (x) was calculated taking the ratio of these high molecular weight fragments from treated DNA (T-DNA) to untreated DNA (C-DNA), based on the Poisson distribution:

$$x = -\ln(\text{T-DNA/C-DNA}).$$

RESULTS

$^1O_2$-induced lesions block *in vitro* DNA synthesis

Primed templates were replicated by DNA polymerases and the products of such reactions were analyzed by electrophoresis. The results obtained when the treated DNA is used as template for sequenase are presented in Figure 1. The clear observation in this figure is the appearance of blocking sites for polymerization in ssDNA treated with $NDPO_2$. Higher doses of $NDPO_2$ (compare lanes 2 and 3) inhibited the synthesis of high molecular weight DNA, due to the presence of an increasing number of blocking sites. The location of these sites was identified by comparison with the *supF* sequence determined on the same gel. The blocking sites for sequenase correspond in general to cytosines in the control sequence, i.e., to dGs in the template DNA. Therefore, DNA damage at the dG residues blocks DNA polymerization *in vitro*.

To ascertain that $^1O_2$ is responsible for the formation of the observed DNA lesions, several control experiments were peformed. No lesions were detected when DNA was treated with NDP, a product of $NDPO_2$ thermolysis (results not shown). When DNA treatment was performed in the presence of NaN_3, there is a significant increase in the amount of high molecular weight DNA being synthesized, probably due to the quenching effect on $^1O_2$ by azide (compare lanes 3 and 4, Figure 1). Altogether, these results suggest that the blocking lesions induced during the thermolysis of $NDPO_2$ are due to the deleterious effects of $^1O_2$ action.

The experiments done allowed the identification of individual bases up to approximately 260 nucleotides from the primer. The relative amount of molecules synthesized with sizes above this limit was quantified by densitometry. The ratio between the values obtained for treated and untreated DNA represents the fraction of molecules which were replicated without finding any blocking lesion induced by $^1O_2$. Based on three different experiments, the number of lesions was then calculated, using the Poisson distribution. It was estimated that about 1.5 lesions per 260 nucleotides were induced after treatment of ssDNA with 5 mM of $NDPO_2$. This value corresponds to about 2% of the guanines present and it is 20 times the amount of breaks in ssDNA induced by treatment with $NDPO_2$ in similar conditions (10). These data imply that most of the $^1O_2$-induced DNA blocking lesions are lesions other than breaks in the phosphodiester chain.

Distribution of the blocking lesions on the *supF* tRNA gene

Several experiments such as those shown in Figure 1 were scanned and the intensity of each individual band was determined at the *supF* gene sequence. The results for two different DNA polymerases are presented in Figure 2. These data correspond to the distribution of blocking sites induced in this sequence by $^1O_2$ and the high specificity of sites close to dG residues in the template can be inferred. In general, sequenase and Klenow fragment present the same distribution pattern. In the experiments shown, different $NDPO_2$ concentrations were used in order to obtain distributions over the entire *supF* sequence for both enzymes. Similar results were obtained for other DNA polymerases (AMV-RT, T4 DNA polymerase and Taq DNA polymerase: not shown), showing that the dGs are equally

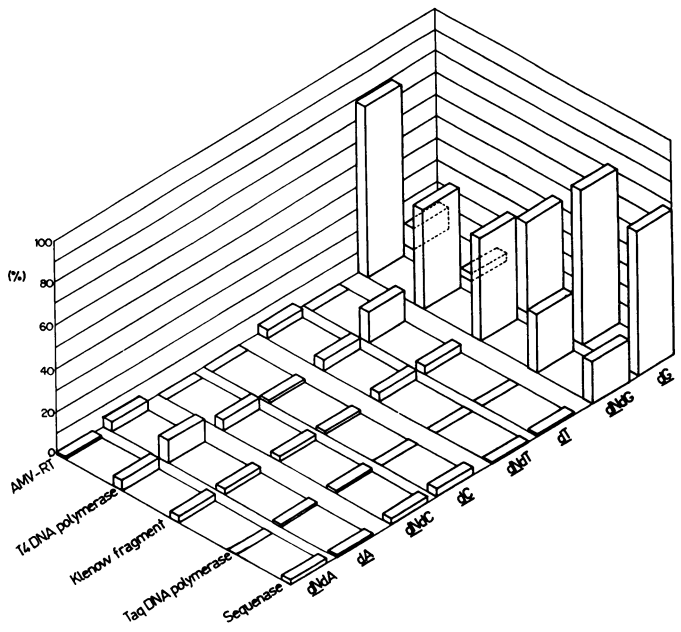

Figure 3. Relative frequencies of the DNA synthesis blocking sites at each base. The data as those shown in Figure 2 were grouped and the relative frequencies of blocking sites either at one base before (dN) or at the base for a given nucleotide were calculated. To minimize the influence of damages at dG's on the data of other bases, the frequencies of dN sites do not include those where dN=dG. For the same reason, blocking sites at a given base are not considered when the nucleotide is before a dG. The sequence analyzed comprises 197 bases for sequenase, 100 bases for Taq DNA polymerase, 182 bases for Klenow fragment, 54 bases for T4 DNA polymerase and 100 bases for AMV-RT.

effective blocks for these enzymes. There are marked variations in the intensity of the bands among the different dGs in the *supF* gene. For example, the dGs at the positions −23, −20 and 1 are not efficient blocking sites, while those at positions −3, 31, 36, 50 and 56 strongly inhibit the elongation of DNA synthesis. The differences in the intensities of such bands may be explained by the preferential $^1O_2$-induction of lesions on different dG's, or by the ability of the DNA polymerases to bypass some dG lesions, depending on the sequence context of the targeted nucleotide. Nevertheless, no particular sequence with special susceptibility to the formation of $^1O_2$-induced damage was detected.

Sites of DNA synthesis interruption by the $^1O_2$-induced lesions for several DNA polymerases

Experiments of DNA synthesis interruption were repeated using five different enzymes. The data obtained for each base in the sequence analyzed were grouped and the relative frequencies of blocking sites before or at each base (dA, dC, dG and T) were determined (Figure 3). It can be seen that most of the DNA synthesis is interrupted opposite to or one base 3′ to a dG, for all the enzymes employed. However, each of these enzymes behaves differently when acting on a $^1O_2$-damaged template. DNA synthesis by sequenase is preferentially blocked opposite to dG residues on the DNA template and 87% of the blocks are located at sites related to this base. Similarly, the Taq DNA polymerase is mainly blocked at dGs, but with almost 98% of the blocks specifically located either before or at this base. DNA synthesis by Klenow fragment is found to be about equally inhibited at and 3′ to dGs (81%), with a small preference for sites preceding dGs. The AMV-RT and the T4 DNA polymerase mainly stop one nucleotide 3′ to dGs on the template. The relative frequency of blocks in positions related to dGs is 90% for AMV-RT and 51% for T4 DNA polymerase. For the latter enzyme, where the frequency of stops apparently unrelated to dGs is high (49%), the synthesis seemed to be blocked two or more bases prior dGs. This result (not shown) together with the high specificity of blocks related to dGs by Taq DNA polymerase suggest that DNA synthesis interruptions not detected at dGs may still be due to lesions at this base.

DISCUSSION

The damaging action of $^1O_2$ on a specific DNA sequence, the *E.coli supF* gene, was studied. The observations gave evidence of the induction of lesions specificallly at dG residues, which interrupt DNA synthesis *in vitro* by several DNA polymerases. The data presented reinforce earlier suggestions that dG is the main target to $^1O_2$ either as free nucleotide (3,4,16) or on DNA (11,12,13,16,22).

The precise nature of these DNA blocking lesions is unknown. Breaks in the template backbone will certainly terminate DNA synthesis. However, single strand breaks correspond to only 5% of the number of blocking lesions (10, and this work). This means that most of the blocking lesions are not breaks in the phosphodiester chain of treated ssDNA.

The induction of 8-OH-dG on DNA treated by photoactivated methylene blue was reported as a possible product of dG oxidation by $^1O_2$ (15). It was found that 8-OH-dG formation by this system exceeds by 17 fold the number of strand breakages (23), a ratio similar to the one estimated here for blocking sites. More recently, Devasagayam et al. (16) showed that exposure of free nucleotides to $NDPO_2$ yields 8-OH-dG as the main product of dG oxidation by $^1O_2$. Therefore, it seems reasonable to suppose that at least part of the blocking sites detected are in fact 8-OH-dG. The ability of such lesions to block DNA synthesis is however a matter of discussion. By using oligonucleotides containing 8-OH-dG as template for DNA synthesis by several DNA polymerases, Shibutani et al. (24) showed that this modified base can transiently interrupt DNA polymerization. This result contrasts with earlier observation of Kuchino et al. (25), who used a similar template and found that this lesion is misread by DNA polymerase I, without retarding DNA synthesis. The experimental procedures used may explain the different results. In the experiments described here whole ssDNA molecules were used, where the effect of 8-OH-dG on DNA polymerization is not known. A more detailed analysis, detecting and quantifying the amount of this lesion on damaged DNA, in conditions similar to those described here is needed.

For the distribution of blocking lesions in the *supF* gene, it can be noted that, in general, dG-rich sequences have stronger blocking sites than isolated dGs. Other than this, no particular pattern of flanking bases was established either in lightly or in highly damaged sites, indicating that the observed differences in the amount of blocking lesions at each dG may be due to more general DNA structural effects on the interaction with $^1O_2$. The role of DNA structure in the formation of $^1O_2$-induced lesions can be inferred by the higher susceptibility of ssDNA in comparison to dsDNA (10).

The observed differences in the behavior of several DNA polymerases when encountering a $^1O_2$-induced damage seem to

be related to the editing activity (3'-5'exonuclease) of each enzyme, as reported before for other kinds of lesion (19,26). In general, enzymes with strong 3'-5'exonuclease activity (Klenow fragment and T4 DNA polymerase) stop preferentially one base before the damaged dG. Enzymes without this activity (sequenase and Taq DNA polymerase) synthesize DNA fragments which are interrupted at the damaged base. The AMV-RT is the only enzyme which does not fit this explanation. DNA polymerization is interrupted one base preceding the putative damaged sites, in spite of the fact that AMV-RT has no 3'-5'exonuclease activity. This result is in agreement with those reported by Larson and Strauss (27), who found that DNA synthesis by AMV-RT is blocked one base before lesions in templates damaged by UV-irradiation, oxiranylpyrene and benzo(*a*)pyrenediol epoxide. Therefore, the 3'-5'exonuclease activity, although important, is not the only feature responsible for the pattern of DNA synthesis termination by a lesion. In fact, several factors, such as the stereochemical properties of the lesion, particular features of each enzyme and the reaction conditions may contribute to the process of polymerization stops on damaged templates.

The experiments described here clearly show that the $^1O_2$-induced lesions at dGs interrupt DNA synthesis *in vitro*. It was previously shown (10) that when treated ssDNA, from the πSVPC13FIA vector, is introduced into mammalian cells, one lethal lesion was induced by 0.5 mM $NDPO_2$, a dose that, according to this work, produces about 3 blocking lesions per plasmid. This means that, *in vivo*, at least two out of three of these lesions are either repaired or bypassed by the replication machinery of these cells. These processes may result in mutations, as those already observed (10). Therefore, the potential deleterious and mutagenic effects of $^1O_2$ on DNA make this excited molecule an important candidate for one of the oxygen reactive species generated in organisms which cause genetic hazards.

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REFERENCES

1. Merkel, P.B. and Kearns, D.R. (1972) *J. Am. Chem. Soc.*, **94**, 7244–7253.
2. Piette, J. (1990) *J. Photochem. Photobiol. B, Biol.*, **4**, 335–342.
3. Cadet, J., Berger, M., Decarroz, C., Wagner, J.R., Van Lier, J.E., Ginot, Y.M. and Vigny, P. (1986) *Biochimie*, **68**, 813–834.
4. Lee, P.C.C. and Rodgers, M.A.J. (1987) *Photochem. Photobiol.*, **45**, 79–86.
5. Boye, E. and Moan, J. (1980) *Photochem. Photobiol.*, **31**, 223–228.
6. Fiel, R.J., Datta-Gupta, N., Mark, E.H. and Howard, J.C. (1981) *Cancer Res.*, **31**, 3543–3545.
7. Blazek, E.R., Peak, J.G. and Peak, M.J. (1989) *Photochem. Photobiol.*, **49**, 607–613.
8. Di Mascio, P., Wefers, H., Do-Thi, H-P., Lafleur, M.V.M. and Sies, H. (1989) *Biochim. Biophys. Acta*, **1007**, 151–157.
9. Di Mascio, P., Menck, C.F.M., Nigro, R.G., Sarasin, A. and Sies, H. (1990) *Photochem. Photobiol.*, **51**, 293–298.
10. Ribeiro, D.T., Madzak, C., Sarasin, A., Di Mascio, P., Sies, H. and Menck, C.F.M. (1992) *Photochem. Photobiol.*, **55**, 39–45.
11. Friedman, T. and Brown, D.M. (1978) *Nuc. Acids Res.*, **5**, 615–622.
12. OhUigin, C., McConnell, D.J., Kelly, J.M. and Van der Putten, J.M. (1987) *Nuc. Acids Res.*, **15**, 7411–7427.
13. Piette, J. and Moore, P.D. (1982) *Photochem. Photobiol.*, **35**, 705–708.
14. Piette, J., Calberg-Bacq, C.M., Lopez, M. and Van de Vorst, A. (1984) *Biochim. Biophys. Acta*, **781**, 257–264.
15. Floyd, R.A., West, M.S., Eneff, K.L. and Schneider, J.E. (1989) *Arch. Biochem. Biophys.*, **273**, 106–111.
16. Devasagayam, T.P.A., Steenken, S., Schultz, W.A. and Sies, H. (1991) *Biochemistry*, **30**, 6283–6289.
17. Di Mascio, P. and Sies, H. (1989) *J. Am. Chem. Soc.*, **111**, 2909–2914.
18. Decuyper-Debergh, D., Piette, J. and Van de Vorst, A. (1987) *EMBO J.*, **10**, 3155–3161.
19. Moore, P.D. and Strauss, B.S. (1979) *Nature*, **278**, 664–666.
20. Yanish-Perron, C., Vieira, J. and Messing, J. (1985) *Gene*, **33**, 103–119.
21. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
22. Kawanishi, S., Inoue, S. and Sano, S. (1986) *J. Biol. Chem.*, **261**, 6090–6095.
23. Schneider, J.E., Price, S., Maidt, L., Gutteridge, J.M.C. and Floyd, R.A. (1990) *Nuc. Acids Res.*, **18**, 631–635.
24. Shibutani, S., Takeshita, M. and Grollman, A.P. (1991) *Nature*, **349**, 431–434.
25. Kuchino, Y., Mori, F., Kasai, H., Inoue, H., Iwai, S., Miura, K., Ohtsuka, E. and Nishimura, S. (1987) *Nature*, **327**, 77–79.
26. Moore, P.D., Bose, K.K., Rabkin, S.D. and Strauss, B.S. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 110–114.
27. Larson, K.L. and Strauss, B.S. (1987) *Biochemistry*, **26**, 2471–2479.